IONIC CONTROL OF SETTLEMENT AND METAMORPHOSIS IN LARVAL *HALIOTIS RUFESCENS* (GASTROPODA)

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ABSTRACT

An increase in the concentration of K^+ in defined sea water medium is demonstrated to induce settlement and metamorphosis in larvae of the marine gastropod mollusc, *Haliotis rufescens*. A decrease in external K^+ ion concentration can inhibit the larval response to γ-aminobutyric acid (GABA), a stereochemically specific inducer of metamorphosis of *H. rufescens*. Stimulation of the metamorphic response by GABA or by increased K^+ may depend on transmembrane movement of ions, since induction is sensitive to neuropharmacological blockers of ion conductance. Sulfonyl isothiocyanostilbene (SITS, an anion exchange blocker) inhibits the larval response to GABA, but does not affect induction by increased external potassium. In contrast, the larval response to potassium is inhibited by tetraethylammonium (TEA, a potassium channel blocker), while induction of metamorphosis by GABA is independent of the presence of TEA. Most manipulations of the concentrations of the other predominant cation components of sea water are not in themselves inductive or inhibitory. However, the actions of GABA and increased K^+ as inducers are sensitive to changes in external Ca^{2+}. Potassium may act by directly depolarizing excitable cells involved in the larval perception of inductive stimuli. Activation of metamorphosis by GABA may depend similarly on a depolarizing ion movement at GABA-sensitive cells. Depolarization by manipulation of the ionic environment may offer a general technique for inducing metamorphosis in various marine invertebrate larvae.

INTRODUCTION

Larval metamorphosis, an essential process in the development of most marine molluscs, is a cascade of complex changes initiated in many cases by specific environmental stimuli (Crisp, 1974; Chia and Rice, 1978). The induction of metamorphosis in larvae of the red abalone, *Haliotis rufescens,* normally depends on the larval encounter of crustose red algae (Morse *et al.,* 1979; 1980c; Morse and Morse, 1984). This inductive action can be mimicked effectively by micromolar concentrations of γ-aminobutyric acid (GABA). When reared at 15°C the planktonic abalone larvae become competent by seven days post-fertilization to respond to the intact alga, algal homogenate, or to micromolar GABA with rapid metamorphosis (Morse *et al.,* 1979, 1980a, b, c). In the continuous presence of an inducer, the larvae cease swimming and attach by the foot to the substrate; this distinct behavioral transition is followed by the characteristic metamorphic sequence described previously (Morse *et al.,* 1980a).

Marine larvae can sense inductive stimuli in the environment, and respond with a coordinated set of behavioral, anatomical, and physiological changes, in a complex process that is likely to involve the larval nervous system (Bonar, 1976; Hadfield, 1978; Burke, 1983a, b). With *Haliotis rufescens,* the direct electrophysiological analysis

of nervous system involvement is handicapped by the small size of the larvae; we have investigated the function of excitable cells instead by manipulation of ion concentrations and the use of neuropharmacological probes. Evidence presented here demonstrates that the induction of metamorphosis in *H. rufescens* is directly affected by changes in the external concentration of potassium, a physiologically important ion capable of driving both hyperpolarizing and depolarizing shifts in cell membrane potential. The pattern of dose-dependent mimicry or inhibition of GABA action by K^+ is predictable by analogy with the observed influence of K^+ on membrane potential in other excitable cell systems. The sensitivity of induction by GABA to changes in external ion concentration, and to specific neuropharmacological probes, suggests that GABA acts similarly as an excitatory agent, producing depolarization of cells capable of activating metamorphosis. Results obtained with neuropharmacological probes suggest that transmembrane movement of specific ions is required for the activation of metamorphosis by increased K^+ or by GABA. These results are consistent with the hypothesis that the depolarization of externally accessible excitable cells alone is sufficient to initiate behavioral and developmental metamorphosis.

MATERIALS AND METHODS

Larval culture

Fertilization was controlled by the mixing of washed gametes, spawned by female and male gravid adult *Haliotis rufescens* after a brief exposure to dilute hydrogen peroxide (Morse *et al.,* 1977). Clean healthy cultures of the veliger larvae, maintained in flowing 5 μm-filtered ultraviolet-irradiated sea water at 15.0 $\pm$ 1.0°C, synchronously developed to a stage of competence to respond to inducers of metamorphosis by seven days post-fertilization (Morse *et al.,* 1980a).

Artificial sea water media

All experiments were conducted in defined sea water media based on the Woods Hole Marine Biological Laboratory (MBL) recipe (Cavanaugh, 1956). Salt and ion concentrations of this medium are summarized for reference in Table I. Ion concentrations were manipulated by modification of the MBL formula in two ways: (a) ion excess, in which addition of a salt to MBL sea water increased concentrations of the selected anionic and cationic species without reducing the concentrations of MBL sea water components; and (b) ion replacement, in which a single ion species was partially or completely replaced with a molar equivalent of ionic charge by another species (without compensation for differences in dissociation constants). Artificial sea water media were made with reagent grade salts volumetrically diluted in glass-distilled and microfiltered (Barnstead Nanopure) water. The final pH values of all normal and modified MBL media ranged from 7.8 to 8.1 without adjustment. Just prior to use, media were innoculated with the antibiotics potassium penicillin G and dihydrostreptomycin sulfate at 150 ppm each, and equilibrated to 15 $\pm$ 1°C.

Assays of induction

All assays were begun with competent veliger larvae (0.2 mm maximum diameter) at 8–10 days post-fertilization. Approximately 200 to 300 larvae were pipetted in a drop of sea water into each 10-ml aliquot of experimental medium, contained in a glass vial (2.4 cm diameter, American Scientific Products). Larvae were incubated in duplicate samples, at 15.0$\pm$1.0°C. Induction of plantigrade attachment, assayed as

The salt composition of MBL sea water medium, taken from Cavanaugh (1956) (A),
and the calculated maximum free ion concentrations (B)

Component	Concentration (mM)
A. *Salt*	
NaCl	423.0
KCl	9.00
CaCl$_2$	9.27
MgCl$_2$	22.94
MgSO$_4$	25.50
NaHCO$_3$	2.15
B. *Ion*	
Na$^+$	425.2
K$^+$	9.00
Ca^{2+}	9.27
Mg^{2+}	48.44
Cl$^-$	496.4
SO$_4^{2-}$	25.50

the percentage of larvae firmly attached by the foot, provided a quantitative measure of the larval metamorphic response as a function of time. Completion of metamorphosis was verified by the abcission of the velum (the larval swimming organ) and the initiation of adult shell growth.

Modified sea waters found to produce toxic effects were disqualified from further analysis. Moderate toxicity was recognized in non-induced or pre-metamorphic larvae by absence of the normal swimming behavior: many larvae remained withdrawn in their shells; ciliary activity was decreased; the few swimming larvae moved feebly through the lower water column or spun slowly in circles against the bottom. Larvae introduced into highly toxic conditions remained withdrawn; the rapid paralysis of ciliary and muscular activity was followed by death.

Neuropharmacological agents tested in conjunction with modified sea water media were added to vials and agitated (Vortex mixer) before temperature equilibration and addition of larvae. γ-Aminobutyric acid (GABA), from Sigma Chemical Company, was used at 4×10^{-7} M, a threshold concentration with which facilitation and inhibition are readily detected. SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate) was obtained from ICN Nutritional Biochemicals, and tetraethylammonium chloride (TEA) from Eastman Kodak Company. Mallinckrodt Chemical Works analytical reagent grade salts were used in the construction of artificial sea water media, with the exception of the highly hygroscopic salt MgCl$_2 \cdot$6H$_2$O which was purchased as a 4.9 M stock solution from Sigma Chemical Company.

RESULTS

The concentration- and time-dependent responses of larvae to GABA in MBL sea water (Fig. 1) are comparable to the responses of larvae in natural sea water, as defined previously (Morse *et al.*, 1979; 1980a). Typically, 40–60% of the larvae display an attachment response to 4×10^{-7} M GABA by 40 h. Although this value ranges between extremes of 30–90% for different cultures, larval responses within a healthy culture are consistent; variation between duplicate vials remains small.
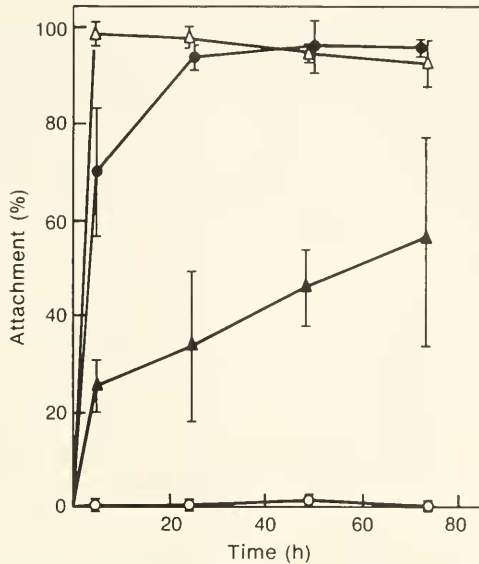
FIGURE 1. Larval attachment in response to GABA in MBL sea water. GABA was added at 10^{-3} M ($\triangle$), 10^{-6} M ($\bullet$), 4×10^{-7} M ($\blacktriangle$), and 0 M ($\bigcirc$). Data are averages of duplicates, with standard deviations indicated by vertical bars.

Ion excess effects

Increased external potassium effectively induced larval attachment, whether added as sulfate or chloride salts to MBL sea water, or used as a replacement for either Na^+ or Mg^{2+} (Fig. 2). In the paired response curves, K^+ added with Cl^- was slightly more efficient as an inducer than when added with SO_4^{2-}. Similarly, the limiting concentration of 20 mM KCl was more rapidly toxic than 10 mM K_2SO_4 (data not included). Both the inductive and toxic effects of K^+ were slightly reduced in medium with sulfate present as the paired anion, instead of chloride.

Increases in the external concentrations of other sea water cations, added in excess to MBL sea water as Cl^- and SO_4^{2-} salts, were not inductive (Table II). With the exception of increased Ca^{2+}, the presence of the various excess salts did not inhibit larval attachment in response to 4×10^{-7} M GABA, indicating that an increase in osmotic pressure alone neither induces nor inhibits induction of metamorphosis. The inhibitory effect of increased Ca^{2+} on induction by GABA corroborates the results obtained from media in which Ca^{2+} concentration was increased by the replacement of Mg^{2+} (as reported below); these results single out Ca^{2+}, rather than concomitant alterations in the substitute or paired salt ion concentration, as the cause of the inhibitory effect.

We have observed that the induction of metamorphosis by excess K^+ is comparable in several respects to that observed with GABA. The efficiency of induction is a dose-dependent function, limited at high concentrations by toxicity. The process of induction involves a temporal component; an optimal concentration of the stimulus (either GABA or increased K^+) must be provided continuously for at least 20 h in order for complete metamorphosis to occur. Premature withdrawal or application of subthreshold levels of the stimulus either fails to induce, or results in only a temporary attachment
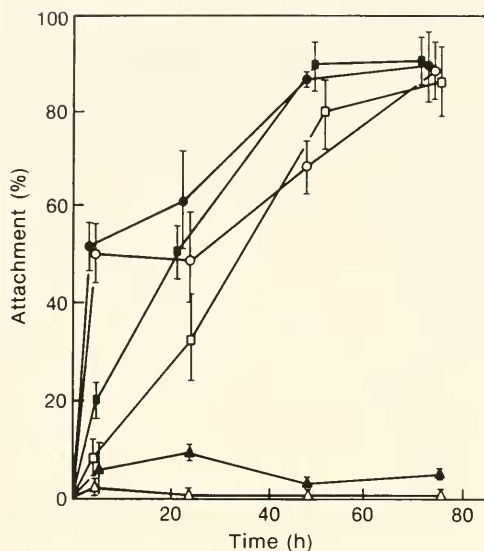
FIGURE 2. Induction of larval attachment by increased external potassium. Potassium was added in excess to MBL sea water as KCl or K_2SO_4 or was used as a replacement for Mg^{2+} or Na^+ in modified MBL sea waters. Excess K^+ was added to MBL sea water as: 2.0 mM K_2SO_4 ($\triangle$), 4.0 mM KCl ($\blacktriangle$), 6.0 mM K_2SO_4 ($\bigcirc$), and 12.0 mM KCl ($\bullet$). The effects of increased K^+ concentrations resulting from replacement were tested with media in which: 5.0 mM Mg^{2+} was replaced with 10.0 mM K^+ ($\square$), and 9.0 mM Na^+ was replaced with 9.0 mM K^+ ($\blacksquare$). Data are averages of duplicates, with standard deviations indicated as vertical bars.

response that is not followed by completion of metamorphosis. Larvae in media with optimal concentrations of increased K^+ or GABA remain active and responsive; premetamorphic larvae swim normally, while attached larvae in the process of metamorphosis crawl actively on the glass substrate, shed velar lobes, and proceed with growth of new adult shell.

Ion replacement effects

Most cations tested as potential substitutes for sea water cations had toxic effects on larvae and were not used. These ions, replacing either Na^+ or K^+ at concentrations of ≤ 9 mM, included Cs^+, Li^+, choline$^+$, Tris$^+$ [tris(hydroxymethyl)amino-methane], and TEA (tetraethylammonium). However, partial replacements of cations with other MBL sea water cations were tolerated well by the larvae, and were used in tests representing a matrix of exchanges (Table III). Data from two consecutive experiments, which used larvae from two separate hatches, were compiled by normalizing the responses to those obtained with GABA (4×10^{-7} M) in unaltered MBL sea water. Each of the four cations present in MBL sea water (Na^+, K^+, Ca^{2+}, Mg^{2+}) was singly replaced by each of the three other species, and the effect of each replacement assayed as a function of time in the presence and absence of 4×10^{-7} M GABA. Response groups presented in Table III show consistent patterns of the effects of the cation exchanges: (Group A) normal induction of metamorphosis by 4×10^{-7} M GABA in unaltered MBL sea water; (Group B) rapid induction of attachment, with or without GABA present, by increased external potassium (except when replacing calcium); (Group C) inhibition of the attachment response to GABA by reduced external po-

TABLE II

Larval attachment responses in MBL sea water media modified by the addition of excess salts (other than potassium)

Ion excess		Larval attachment (% ± S.D.)	
Salt	Conc. (mM)	−GABA	+GABA[1]
None	(MBL sea water)	0 ± 0	72 ± 5
NaCl	10	0 ± 0	52 ± 10
	20	1 ± 0	60 ± 6
	40	1 ± 1	65 ± 6
Na_2SO_4	10	1 ± 1	54 ± 1
	20	2 ± 1	66 ± 10
	40	8 ± 3	64 ± 3
[2]$CaSO_4$	10	2 ± 0	45 ± 21
	20	2 ± 1	35 ± 3
[2]$CaCl_2$	10	0 ± 0	69 ± 11
	20	1 ± 1	28 ± 7
$MgCl_2$	10	0 ± 0	69 ± 13
	20	0 ± 0	66 ± 8
	40	0 ± 0	40 ± 13

[1] Attachment responses are shown at 47 h exposure; GABA was used at 4×10^{-7} M.
[2] Calcium salts at 40 mM were toxic (as defined in text); larval attachment in these media +GABA was ≤1%.

tassium (except when replaced by magnesium); (Group D) induction, without GABA present, by medium in which sodium was used to replace magnesium, and conversely, inhibition of GABA-induced attachment by medium in which magnesium was replaced by sodium; (Group E) inhibition of the attachment response to GABA by increased external calcium; (Group F) absence of inductive, facilitative, or inhibitory effects of media, without GABA present, in which external calcium concentration was reduced. The absence of inductive ability of increased external K^+ when replacing Ca^{2+} is unique to that exchange condition.

In contrast, decreased potassium (Table III, Group C) was not inductive in any exchange condition. When K^+ was partially replaced by 5.0 mM Na^+ or 2.5 mM Ca^{2+}, inhibition of GABA action was observed, although increased Ca^{2+} itself also produced inhibition (Group E). The result with substitution by Na^+, however, indicates that decreased external K^+ can inhibit the larval response to GABA.

The only cation replacement capable of inducing attachment of larvae without GABA present, other than replacements resulting in an increase of external potassium, was that in which external Mg^{2+} was replaced with Na^+. The substitution of 23.0 mM Mg^{2+} with 46.0 mM Na^+ (which permitted the concentration of the paired anion to remain unchanged) was inductive at a level comparable to that of 4×10^{-7} M GABA (Table III, Group C). A comparison with the other substitution conditions in Table III in which Mg^{2+} was decreased, or in which Na^+ was increased, indicates that neither ion shift alone can be credited with the inductive action. Apparently, it is the specific replacement of Mg^{2+} with Na^+ that effects larval attachment. The reverse replacement of 46.0 mM Na^+ with 23.0 mM Mg^{2+} strongly inhibited induction by 4×10^{-7} M GABA. Again, a comparison of the results obtained with other media in which external Na^+ was decreased, or Mg^{2+} was increased, shows that neither cation change alone is consistently inhibitory.

TABLE III

Larval attachment responses in MBL sea water media modified by cation replacement

	Replacement media				Larval response[1]			
					−GABA		+GABA	
Group	Cation replaced	Conc. (mM)	Cation substituted	Conc. (mM)	Relative attachment (% ± S.D.)[2]	Effect[3]	Relative attachment (% ± S.D.)[2]	Effect[3]
A	(MBL sea water control; no replacement)				0 ± 0.0	N	100 ± 8.4	N
B	Na⁺	9	K⁺	9	143 ± 5.6	I	148 ± 2.8	F
	Na⁺	18	K⁺	18	152 ± 7.0	I	143 ± 7.0	F
	Mg²⁺	5	K⁺	10	159 ± 4.2	I	158 ± 1.4	F
	Ca²⁺	5	K⁺	10	0 ± 0.0	N	101 ± 1.4	N
C	K⁺	5	Na⁺	5	0 ± 0.0	N	61 ± 13	X
	K⁺	5	Mg²⁺	2.5	0 ± 0.0	N	92 ± 21	N
	K⁺	5	Ca²⁺	2.5	0 ± 0.0	N	59 ± 4.2	X
D	Mg²⁺	23	Na⁺	46	97 ± 9.8	I	117 ± 1.4	N
	Na⁺	46	Mg²⁺	23	0 ± 0.0	N	10 ± 2.8	X
E	Mg²⁺	23	Ca²⁺	23	4 ± 1.4	N	62 ± 2.8	X
	Na⁺	23	Ca²⁺	11.5	0 ± 0.0	N	46 ± 7.0	X
	⁴K⁺	5	Ca²⁺	2.5	0 ± 0.0	N	59 ± 4.2	X
F	Ca²⁺	5	Na⁺	10	0 ± 0.0	N	92 ± 11	N
	Ca²⁺	5	Mg²⁺	5	0 ± 0.0	N	89 ± 1.4	N
	⁴Ca²⁺	5	K⁺	10	0 ± 0.0	N	101 ± 1.4	N

[1] Attachment responses are shown at 45 h exposure; GABA was used at 4×10^{-7} *M*.

[2] Normalized to attachment observed in MBL sea water + GABA, as explained in Results; the absolute value of attachment in MBL seawater + GABA was 47%. S.D. is the absolute standard deviation.

[3] Effects: (N) no facilitating or inhibitory effect compared with response in unmodified MBL sea water; (I) induction without GABA; (F) facilitation of induction by GABA; (X) inhibition of induction by GABA.

[4] Media listed twice for comparison in separate groups.

TABLE IV

The effects of altered concentrations of external Ca^{2+} on larval attachment responses to GABA and to increased K^+

				Larval attachment (% ± S.D.)					
Altered ion concentrations[1]				−GABA			+GABA[2]		
K⁺	Na⁺	Ca²⁺	Mg²⁺	22 h	49 h	72 h	22 h	49 h	72 h
None (MBL sea water)				0 ± 0	1 ± 1	0 ± 0	60 ± 13	80 ± 21	89 ± 4
		+4	−4	0 ± 0	0 ± 0	0 ± 0	36 ± 2	88 ± 6	94 ± 0
		+9	−9	0 ± 0	1 ± 1	0 ± 0	11 ± 6	41 ± 3	52 ± 13
+12	−12			62 ± 12	57 ± 3	74 ± 1	79 ± 3	90 ± 3	94 ± 1
+12	−12	+4	−4	63 ± 3	80 ± 4	93 ± 3	75 ± 0	95 ± 1	96 ± 3
+12	−12	+9	−9	57 ± 12	81 ± 1	96 ± 3	64 ± 16	83 ± 9	95 ± 4
		−4	+4	0 ± 0	1 ± 1	0 ± 0	41 ± 17	69 ± 20	95 ± 2
		−9	+9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 0	0 ± 0
+12	−12	−4	+4	5 ± 2	4 ± 3	2 ± 2	72 ± 2	82 ± 0	87 ± 2
+12	−12	−9	+9	2 ± 1	0 ± 0	0 ± 0	9 ± 1	43 ± 13	44 ± 10

[1] Changes in cation concentration (mM) with reference to standard MBL sea water.

[2] GABA was used at 4×10^{-7} *M*.

The actions of GABA and increased external K^+ as inducers of metamorphosis both were inhibited by changes in external Ca^{2+}, although the directions of the net change in Ca^{2+} to which they were sensitive were opposite (Table IV). Without an inducer present, the changes in external Ca^{2+} (imposed in combination with reciprocal equimolar changes in external Mg^{2+}) had no effect on exposed larvae. Larval attachment in response to 4×10^{-7} M GABA was inhibited by a 9.0 mM increase in Ca^{2+}. Larval responses to increased external K^+ (introduced as an equimolar replacement for Na^+, without GABA present) were not affected by increased external Ca^{2+}, indicating that inhibition of the response to GABA was not caused by toxicity. In contrast, the larval response to GABA in medium with Ca^{2+} decreased by 4.0 mM remained comparable to that in MBL sea water with GABA. However, the larval response to increased K^+ was strongly inhibited by the 4.0 mM reduction in Ca^{2+}. Despite this strong inhibition, the normal response of larvae to GABA (present in addition to the increased K^+ and decreased Ca^{2+}) was retained, again negating the possibility that toxicity was the cause of inhibition. Virtually complete replacement of Ca^{2+} (-9.0 mM) inhibited attachment in all conditions, suggesting that this extreme reduction in external Ca^{2+} was detrimental to the larvae.

Neuropharmacological analyses

Neuropharmacological probes were used to analyze the effects of external ion changes in the initiation of metamorphosis. Induction by GABA is sensitive specifically to the presence of SITS, an isothiocyanate derivative known to inhibit anion exchange (Cabantchik and Rothstein, 1972). Addition of 1×10^{-5} M SITS to MBL sea water inhibited larval attachment in response to GABA, without altering larval behavior in the absence of GABA (Table V). In contrast, the induction of metamorphosis by increased potassium was not affected by SITS. The presence of SITS did not substantially reduce the increase in larval attachment contributed by GABA, when present in addition to 12 mM excess K^+.

The effectiveness of SITS as an inhibitor of GABA action depends on its concentration relative to that of GABA (Fig. 3). SITS at 10^{-4} M fully blocked the inductive effect of 10^{-4} M GABA. A concentration of SITS lower by one order of magnitude (10^{-5} M) did not block induction by 10^{-4} M GABA but did affect the rate of attachment induced by lower concentrations of GABA. SITS at 10^{-6} M was relatively ineffective,

TABLE V

The effects of a K^+-channel blocker (TEA) and an anion exchange blocker (SITS) on larval attachment responses to increased K^+ and GABA

KCl Excess[1]	GABA	Larval attachment (% ± S.D.)[2]		
		Alone	+TEA	+SITS
0	0	0 ± 0	0 ± 0	0 ± 0
	4×10^{-7} M	43 ± 10	47 ± 18	16 ± 1
12 mM	0	54 ± 10	24 ± 11	57 ± 7
	4×10^{-7} M	82 ± 6	43 ± 3	75 ± 0

[1] MBL sea water media prepared as described in text.
[2] Absolute percentage of larvae attached after 24 h exposure; S.D. is standard deviation. Concentrations of additions: TEA (5×10^{-5} M); SITS (1×10^{-5} M).
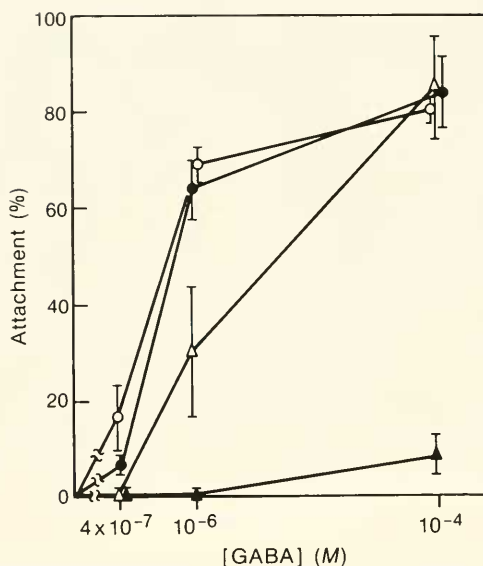
FIGURE 3. Inhibition by SITS, as a function of the relative concentrations of SITS and GABA. GABA concentrations are indicated on the horizontal axis. Larval attachment at 28 h in response to GABA is shown for media in which SITS is present at concentrations of: 10^{-4} M (▲), 10^{-5} M (△), 10^{-6} M (●), and 0 M (○). Data are averages of duplicates, with standard deviations indicated as vertical bars.

except when present with the threshold concentration of 4×10^{-7} M GABA. SITS at concentrations lower than those of GABA seemed to have little inhibitory influence.

At the erythrocyte membrane, the covalent binding of isothiocyanate groups to the anion transporter protein occurs at specific amino groups (Passow *et al.,* 1982). The possibility that SITS might inhibit the larval response to GABA by binding the γ-amino group of GABA and thus decreasing the effective concentration, rather than by acting at larval membrane sites, was tested using glycine, a non-inductive and non-facilitating structural analog. Glycine, added with SITS for a one hour preincubation prior to the addition of GABA and competent larvae, remained continuously present during the subsequent assays of induction. No competitive protection of induction by GABA from inhibition by SITS was evident in the presence of glycine; SITS fully retained its ability to inhibit GABA action (Fig. 4). Glycine alone had no effect on induction by GABA. The possibility that SITS might act to bind GABA, but not glycine, because of steric hindrance of the amino group in the shorter molecule, was tested by repeating the protocol with ε-aminocaproic acid (a longer homolog of GABA) instead of glycine; the identical result further shows that SITS does not act by binding nonspecifically to the amino groups of amino acids in solution. The inhibitory action of SITS appears to be relatively specific. Other potential blockers of ion conductance that were found to have no effect on the normal larval response to GABA include: (a) tetrodotoxin, a blocker of voltage-regulated sodium channels in axonal membranes (review by Armstrong, 1974); (b) picrotoxin, a blocker of GABA-regulated increases in Cl^- permeability in some systems (Takeuchi, 1976; Gallagher *et al.,* 1978; Yarowsky and Carpenter, 1978); and (c) furosemide, an inhibitor of mediated cotransport (Geck *et al.,* 1980).

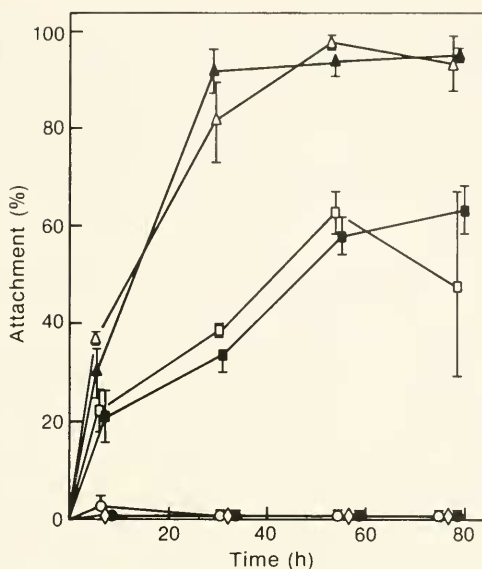The action of potassium in the induction of metamorphosis was analyzed using

FIGURE 4. The inhibitory action of SITS on the larval response to GABA, with or without preincubation of SITS with glycine. SITS and/or glycine, where indicated, were added 1 h before initiation of the experimental assay by addition of GABA, where indicated, and subsequent introduction of competent larvae. Larval responses are shown for MBL sea water with: no addition (○); glycine (●); GABA (△); GABA and glycine (▲); SITS (◇); SITS and GABA (□); SITS and GABA with glycine (■). Concentrations were: GABA 4×10^{-7} M; glycine, 10^{-5} M; and SITS, 10^{-5} M. Data are averages of duplicates, with standard deviations indicated as vertical bars.

tetraethylammonium chloride (TEA), an impermeant blocker of K^+ channels in nerve and muscle cells (review by Armstrong, 1974); both intracellular and extracellular applications of TEA block the Ca^{2+}-activated K^+ current in molluscan neurons (Hermann and Gorman, 1981). At concentrations less than 10^{-4} M, TEA specifically inhibits induction of $H.$ $rufescens$ by increased K^+; higher concentrations of TEA are toxic, and cause non-specific inhibition of larval responses to all inducers. Concentrations of TEA less than 10^{-5} M have no apparent inhibitory effect. The presence of 5×10^{-5} M TEA reduced the inductive action of 12 mM excess KCl and negated the additive effect of increased K^+ when present in combination with 4×10^{-7} M GABA, reducing the attachment to a level equivalent to that of GABA in MBL sea water alone (Table V). Induction by GABA in MBL sea water was unaffected by the presence of TEA at 5×10^{-5}, indicating that inhibition by TEA does not result from toxicity. Function of the TEA-sensitive sites thus is required for the induction of metamorphosis by increased K^+, but apparently is not essential for the pathway activated by GABA.

DISCUSSION

The complete process of metamorphosis is induced in $Haliotis$ $rufescens$ larvae by an increase in the concentration of K^+ in sea water. Changes in external K^+ concentration can drive electrogenic movements of K^+ that directly affect the membrane potential; the depolarization of membrane potential as a function of increasing extracellular K^+ has been used to demonstrate that the excitable membrane can behave in a classical sense as a K^+ electrode (Hodgkin and Horowicz, 1959). The

inductive action of increased K^+ suggests that metamorphosis in *H. rufescens* can be initiated solely by the depolarization of externally accessible excitable cells. Depolarizing electrical stimuli, delivered by suction electrode to the region of the oral ganglion or apical neuropile, have been shown by Burke (1983a) to elicit immediate metamorphosis in competent larvae of the Pacific sand dollar *Dendraster excentricus.* This site-specific efficacy suggests that the metamorphic response to an appropriate environmental stimulus is activated in this species by the neural communication of sensory receptors with the larval nervous system.

The induction of metamorphosis of *H. rufescens* by GABA may depend similarly on the depolarization of GABA-sensitive cells. The initial larval response to GABA in the presence of increased external K^+ is greater than that observed with either GABA or increased K^+ alone (Table V). In contrast to this combined effect of increased K^+ with GABA, a decrease in external K^+ can inhibit induction by GABA. Hyperpolarization resulting from the decrease in external potassium, as demonstrated for GABA-regulated postsynaptic cells (Motokizawa *et al.,* 1969), could antagonize a GABA-mediated depolarization. It is unlikely that GABA acts directly by altering membrane permeability to K^+ at the same sites utilized during induction by increased K^+, since the actions of these inducers are pharmacologically separable. Induction by GABA is sensitive to SITS, and insensitive to TEA; induction by increased K^+ is inhibited by TEA but not by SITS. These reciprocal sensitivities also indicate that the inducers operate through pathways that initially are separate; that is, neither follows the other in an obligatory sequence in the process of induction.

The separateness of the inductive actions of GABA and increased K^+ also is evident in their entirely different sensitivities to alterations in external Ca^{2+}. Induction by GABA is inhibited specifically by increased Ca^{2+}, while induction by increased K^+ is sensitive only to a reduced external concentration of Ca^{2+}. A simple model can be proposed, analogous to other systems, which invokes a single mechanism to explain the opposite sensitivities of these inducers. Increased cytoplasmic concentrations of Ca^{2+} have been shown to activate K^+ conductance through Ca^{2+}-regulated K^+ channels in diverse cell types (review by Schwarz and Passow, 1983). At physiological concentrations of internal and external K^+, a Ca^{2+}-activated increase in K^+ conductance permits a net K^+ efflux that can hyperpolarize a sensory receptor cell, thus decreasing the rate of afferent discharge (review by Edwards, 1984). If we postulate the existence, in larval *H. rufescens,* of Ca^{2+}-regulated K^+ channels in cells that are capable of responding to GABA and to K^+, then the effects of Ca^{2+} can be explained by a comparable mechanism. In medium with a standard sea water concentration of K^+, an increase in calcium (suggested to produce a parallel increase in cytoplasmic calcium) may inhibit the effect of GABA by activating a hyperpolarizing net K^+ efflux. With an inductive increase in external K^+, however, membrane depolarization rather than hyperpolarization would be expected in response to increased Ca^{2+}; this prediction is supported by the observed absence of an inhibitory effect of increased Ca^{2+} on induction by K^+. In contrast, a decrease in external Ca^{2+} (suggested to produce a decrease in cytoplasmic Ca^{2+}) may block induction by K^+ by antagonizing the necessary electrogenic influx of the cation through Ca^{2+}-regulated membrane channels. The reduced efficiency of K^+ as an inducer, when added with sulfate rather than chloride to MBL sea water, may result from a decrease in the sea water concentration of free Ca^{2+}, since $CaSO_4$ has a higher association constant than $CaCl_2$. The induction of metamorphosis by GABA is not sensitive to decreased Ca^{2+}, suggesting that its action is not impaired by an increased membrane resistance to K^+; this idea is supported by our demonstration that induction by GABA is insensitive to the presence of the K^+-channel blocker, TEA. Although a heterogeneous population of larval cells is exposed during the test of an altered sea water medium, the resulting

effects on larval metamorphosis are consistent with this model based on the exclusive function of a single group of accessible excitable cells.

The selective movement of ions across specialized membranes is a fundamental mechanism in the function of excitable cells. At invertebrate chemoreceptors, a stimulus-dependent increase in ion permeability can transduce chemical stimuli into electrical impulses, allowing nervous system analysis of environmental information (Morita, 1972; Thurm and Wessel, 1979; Kaissling and Thorson, 1980). Postsynaptic cells mediate the effect of a chemical neurotransmitter similarly by altering membrane permeability to ions capable of influencing the membrane potential (Takeuchi and Takeuchi, 1960). GABA, as an inhibitory neurotransmitter in both vertebrate and invertebrate systems, acts at postsynaptic sites to increase membrane permeability to chloride (Krnjević and Schwartz, 1967; Takeuchi et al., 1978; review by Takeuchi, 1976). While the inhibitory effect of GABA commonly depends on a hyperpolarizing Cl^- influx, GABA also has been shown to activate a depolarizing efflux of Cl^- in the presynaptic inhibition of vertebrate spinal ganglia (Nishi et al., 1974; Gallagher et al., 1978). GABA can hyperpolarize or depolarize different cells within the same ganglion in invertebrates such as Helix (Walker et al., 1975) and Cancer (Marder and Paupardin-Tritsch, 1978).

The site of action of exogenous GABA as an inducer of metamorphosis of H. rufescens larvae remains unknown. If acting through the larval nervous system, GABA is likely to function either as a ligand mimicking the active component of the inductive algae at larval chemoreceptors, or as a neurotransmitter at synapses between neurons regulating the initiation of metamorphosis. We have shown that the larval response to GABA depends on the function of a SITS-sensitive process. This requirement appears to be specific, since induction by increased K^+ is not inhibited by SITS, and potential blockers of other ion conductances fail to inhibit the larval response to GABA. It is possible that the larval response to GABA may be directly dependent on a GABA-controlled alteration of SITS-sensitive anion exchange. Although anion exchange processes generally are considered to be electrically neutral, GABA could generate the depolarizing net efflux of an anion such as Cl^- by promoting "slippage," an exchanger-mediated process in which the unidirectional transport of an anion occurs without an associated anion countertransport (Knauf et al., 1977; Fröhlich et al., 1983). Alternatively, the SITS-sensitive system may not be directly controlled by GABA, but may be capable of influencing a state or process on which GABA action does depend.

Other data support the suggestion that there may be a functional relationship between GABA as an inducer of metamorphosis, and the transmembrane movement of anions. We have found that the induction of metamorphosis of H. rufescens larvae by GABA is sensitive to changes in Cl^- concentration in artificial sea water. Furthermore, without GABA present, the replacement of 25–75% of Cl^- in sea water with substitute anions (Br^-, SO_4^{2-}, NO_3^-, acetate, isethionate, or propionate) induces attachment of competent larvae; this inductive action is inhibited by SITS, but not by TEA. The macrocyclic lactone ivermectin, a compound demonstrated to increase Cl^- conductance at a GABA-regulated synapse in lobster (Fritz et al., 1979), is inductive alone and facilitates induction by media in which Cl^- is replaced with a substitute anion. An increase in external Cl^-, added in excess with Mg^{2+}, blocks induction by GABA. These results, suggesting that Cl^- efflux may play a role in transduction of the GABA signal, will be presented in more detail elsewhere (Baloun and Morse, in prep.).

The data presented here support the idea that GABA and increased K^+ work similarly by causing depolarization, but require the function of different ion-conductive processes in the induction of metamorphosis. Additional work will be required to

determine how the specific exchanges of Mg^{2+} and Na^+ either induce attachment of larvae or block the response to GABA. The induction of larval attachment by medium in which Mg^{2+} is replaced with Na^+ is insensitive both to SITS and TEA, suggesting a mechanism of action separate from those of GABA and increased K^+.

The culture of marine invertebrates, for research in ontogeny and neurobiology, and for production of food and other resources, would benefit from the development of a general technique for initiating larval metamorphosis. The nature of the specific inducing signals naturally required for metamorphosis is likely to vary among species recruited to different specialized microenvironments. In contrast, the transduction of chemical or other stimuli by receptor cell depolarization may be a more general mechanism in initiating the metamorphic response. The depolarizing effect of small increases in external K^+ thus may provide a simple and economical method for the induction of metamorphosis in a variety of marine invertebrates. This idea is supported by our recent finding that larvae of the gastropod mollusc *Astraea undosa,* for which the natural inducer is not yet identified, efficiently are induced to settle and metamorphose in a dose-dependent response to increased external potassium (Markell, Baloun, and Morse, unpubl. obs.). The optimal concentration of excess potassium required for the metamorphic response of *A. undosa* is close to that described here for *Haliotis.*

While the specific physical and chemical characteristics of substrates influencing settlement of marine invertebrate larvae have been extensively reviewed (Crisp, 1974; Scheltema, 1974; Hadfield, 1978), few studies are available on the role of neurophysiologically important ions in larval induction. Early work by Lynch (1947) suggested that influx of Na^+ during brief exposure to greater than normal concentrations of sodium salts could accelerate metamorphosis of *Bugula* larvae. Spindler and Müller (1972) demonstrated an inductive response to LiCl in planula larvae of *Hydractinia echinata.* Subsequent work by Müller and Buchal (1973) defined a range of inductive responses to Cs^+, Rb^+, Li^+, and K^+. Succinyl choline chloride was shown to induce metamorphosis in *Phestilla* larvae (Bonar, 1976); the active component choline is inductive alone, although less efficient than the natural inducer of metamorphosis (Hadfield, 1978). In these and related studies, the mechanisms of action of the inductive ion changes have remained hypothetical (Müller and Buchal, 1973), or were considered to be unrelated to the normal physiological mechanism (Crisp, 1974, 1984; Hadfield, 1978, 1984). Our results with larvae of *Haliotis rufescens* suggest that these results obtained in other systems, once considered to be artifactual, may in retrospect be recognized as clues to the integral role of ions in transducing the environmental stimuli required for metamorphosis.

LITERATURE CITED

ARMSTRONG, C. M. 1974. Ionic pores, gates, and gating currents. *Q. Rev. Biophys.* **7**: 179–210.

BONAR, D. B. 1976. Molluscan metamorphosis: A study in tissue transformation. *Am. Zool.* **16**: 573–591.

BURKE, R. D. 1983a. Neural control of metamorphosis in *Dendraster excentricus. Biol. Bull.* **164**: 176–188.

BURKE, R. D. 1983b. The induction of marine invertebrate larvae: stimulus and response. *Can. J. Zool.* **61**: 1701–1719.

CABANTCHIK, Z. I., AND A. ROTHSTEIN. 1972. The nature of membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Memb. Biol.* **10**: 311–330.

CAVANAUGH, G. M. 1956. Pp. 62–69 in *Formulae and Methods IV of the Marine Biological Laboratory Chemical Room.* Woods Hole, Massachusetts.

CHIA, F. S., AND M. E. RICE, eds. 1978. *Settlement and Metamorphosis of Marine Invertebrate Larvae.* Elsevier, New York.

CRISP, D. J. 1974. Factors influencing settlement of marine invertebrate larvae. Pp. 177–265 in *Chemoreception in Marine Organisms,* P. T. Grant and A. M. Mackie, eds. Academic Press, New York.

CRISP, D. J. 1984. Overview of research on marine invertebrate larvae, 1940–1980. Pp. 103–126 in *Marine Biodeterioration,* J. D. Costlow and R. C. Tipper, eds. Naval Institute Press, Annapolis, Maryland.

EDWARDS, C. 1983. The ionic mechanisms underlaying the receptor potential in mechanoreceptors. Pp. 497–503 in *The Physiology of Excitable Cells,* A. D. Grinnell and W. J. Moody Jr., eds. Alan R. Liss, Inc., New York.

FRITZ, L. C., C. C. WANG, AND A. GORIO. 1979. Avermectin B_1a irreversibly blocks postsynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance. *Proc. Natl. Acad. Sci. USA* **76**(4): 2062–2066.

FRÖHLICH, O., C. LEIBSON, AND R. B. GUNN. 1983. Chloride net efflux from intact erythrocytes under slippage conditions: Evidence for a positive charge on the anion binding/transport site. *J. Gen. Physiol.* **81**: 127–152.

GALLAGHER, J. P., H. HIGASHI, AND S. NISHI. 1978. Characterization and ionic basis of GABA-induced depolarizations recorded "in vitro" from cat primary afferent neurones. *J. Physiol.* **275**: 263–282.

GECK, P., C. PIETRTYK, B. C. BURCKIIARDT, B. PFEIFFER, AND E. HEINZ. 1980. Electrically silent cotransport of Na^+, K^+ and Cl^- in Ehrlich cells. *Biochim. Biophys. Acta* **600**: 432–447.

HADFIELD, M. G. 1978. Metamorphosis in marine molluscan larvae: an analysis of stimulus and response. Pp. 165–175 in *Settlement and Metamorphosis of Marine Invertebrate Larvae,* F. S. Chia and M. E. Rice, eds. Elsevier, New York.

HADFIELD, M. G. 1984. Settlement requirements of molluscan larvae: New data on chemical and genetic roles. In *Recent Innovations in Cultivation of Pacific Molluscs,* D. Morse, K. Chew, and R. Mann, eds. Elsevier, New York. (In press.)

HERMANN, A., AND A. L. F. GORMAN. 1981. Effects of tetraethylammonium on potassium currents in a molluscan neuron. *J. Gen. Physiol.* **78**: 87–110.

HODGKIN, A. L., AND P. HOROWICZ. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**: 127–160.

KAISSLING, K. E., AND J. THORSON. 1980. Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. Pp. 261–282 in *Receptors for Neurotransmitters. Hormones and Pheromones in Insects,* D. B. Satelle *et al.,* eds. Elsevier/North-Holland Biomedical Press, New York.

KNAUF, P. A., G. F. FUHRMANN, S. ROTHSTEIN, AND A. ROTHSTEIN. (1977). The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69**: 363–386.

KRNJEVIĆ, K., AND S. SCHWARTZ. 1967. The action of γ-aminobutyric acid on cortical neurones. *Exp. Brain Res.* **3**: 320–336.

LYNCH, W. F. 1947. The behavior and metamorphosis of the larva of *Bugula neritina* (Linnaeus): Experimental modification of the length of the free-swimming period and the responses of the larvae to light and gravity. *Biol. Bull.* **92**: 115–150.

MARDER, E., AND D. PAUPARDIN-TRITSCH. 1978. The pharmacological properties of some crustacean neuronal acetylcholine, γ-aminobutyric acid, and L-glutamate responses. *J. Physiol.* **280**: 213–236.

MORITA, H. 1972. Primary processes of insect chemoreception. *Adv. Biophys.* **3**: 161–198.

MORSE, A., AND D. E. MORSE. 1984. Recruitment and metamorphosis of *Haliotis* larvae are induced by molecules uniquely available at the surfaces of crustose red algae. *J. Exp. Mar. Biol. Ecol.* **75**: 191–215.

MORSE, D. E., H. DUNCAN, N. HOOKER, AND A. MORSE. 1977. Hydrogen peroxide induces spawning in molluscs, with activation of prostaglandin endoperoxide synthetase. *Science* **196**: 298–300.

MORSE, D. E., N. HOOKER, H. DUNCAN, AND L. JENSEN. 1979. γ-Aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. *Science* **204**: 407–410.

MORSE, D. E., H. DUNCAN, N. HOOKER, A. BALOUN, AND G. YOUNG. 1980a. GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae. *Fed. Proc.* **39**: 3237–3241.

MORSE, D. E., N. HOOKER, AND H. DUNCAN. 1980b. GABA induces metamorphosis in *Haliotis*, V: Stereochemical specificity. *Brain Res. Bull.* 5, Suppl. **2**: 381–387.

MORSE, D. E., M. TEGNER, H. DUNCAN, N. HOOKER, G. TREVELYAN, AND A. CAMERON. 1980c. Induction of settling and metamorphosis of planktonic molluscan larvae III: Signaling by metabolites of intact algae is dependent on contact. Pp. 67–86 in *Chemical Signaling in Vertebrate and Aquatic Animals,* D. Müller-Schwarze and R. M. Silverstein, eds. Plenum Press, New York.

MOTOKIZAWA, F., J. P. REUBEN, AND H. GRUNDFEST. 1969. Ionic permeability of the inhibitory postsynaptic membrane of lobster muscle fibers. *J. Gen. Physiol.* **54**: 437–461.

MÜLLER, W. A., AND G. BUCHAL. 1973. Metamorphose-induktion bei Planulalarven. II. Induktion durch monovalente Kationen: Die Bedeutung des Gibbs-Donnan-Verhaltnisses und der Na$^+$/K$^+$-ATPase. *Wilhelm Roux' Arch.* **174**: 122–135.

NISHI, S., S. MINOTA, AND A. G. KARCZMAR. 1974. Primary afferent neurones: The ionic mechanism of GABA-mediated depolarization. *Neuropharmacology* **13**: 215–219.

PASSOW, H., H. FASOLD, M. L. JENNINGS, AND S. LEPKE. 1982. The study of the anion transport protein ("band 3 protein") in the red blood cell membrane by means of tritiated 4,4'-diisothiocyano-dihydrostilbene-2,2'-disulfonic acid (^{3}H$_2$-DIDS). Pp. 1–31 in *Chloride Transport in Biological Membranes,* J. A. Zadunaisky, ed. Academic Press, New York.

SCHELTEMA, R. S. 1974. Biological interactions determining larval settlement of marine invertebrates. *Thallasia Jugoslav.* **10**: 263–296.

SCHWARZ, W., AND H. PASSOW. 1983. Ca^{2+}-activated K$^+$ channels in erythrocytes and excitable cells. *Ann. Rev. Physiol.* **45**: 359–374.

SPINDLER, K-D., AND W. A. MÜLLER. 1972. Induction of metamorphosis by bacteria and by a lithium-pulse in the larvae of *Hydractinia echinata* (Hydrozoa). *Wilhelm Roux' Arch.* **169**: 271–280.

TAKEUCHI, A. 1976. Studies of inhibitory effects of GABA in invertebrate nervous systems. Pp. 255–267 in *GABA in Nervous System Function,* E. Roberts, T. N. Chase, D. B. Tower, eds. Raven Press, New York.

TAKEUCHI, A., AND N. TAKEUCHI. 1960. On the permeability of the end-plate membrane during the action of the transmitter. *J. Physiol. Lond.* **154**: 52–67.

TAKEUCHI, H., K. WATANABE, AND H. TAMURA. 1978. Penetrable and impenetrable anions into the GABA-activated chloride channel on the postsynaptic neuromembrane of an identifiable giant neurone of an African giant snail (*Achatina fulica* Ferussac). *Comp. Biochem. Physiol.* **61**C: 309–315.

THURM, U., AND G. WESSEL. 1979. Metabolism-dependent transepithelial potential differences at epidermal receptors of arthropods. *J. Comp. Physiol.* **134**A: 119–130.

WALKER, R. J., M. J. AZANZA, G. A. KERKUT, AND G. N. WOODRUFF. 1975. The action of γ-aminobutyric acid (GABA) and related compounds on two identifiable neurones in the brain of the snail *Helix aspera. Comp. Biochem. Physiol.* **50**C: 147–154.

YAROWSKY, P. J., AND D. O. CARPENTER. 1978. A comparison of similar ionic responses to γ-aminobutyric acid and acetylcholine. *J. Neurophysiol.* **41**: 531–541.